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PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/GB93/01223 (22) International Filing Date: 9 June 1993 (09.06.93) (30) Priority data: 9212164.9 9 June 1992 (09.06.92) GB (71) Applicant (for all designated States except US): MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB). (72) Inventor; and (75) Inventor/Applicant (for US only): HAWKINS, Trevor, Leonard [GB/US]; Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711-5399 (US). (74) Agent: KEITH W. NASH & CO.; Pearl Assurance House, 90-92 Regent Street, Cambridge CB2 1DP (GB).	(81) Designated States: AU, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: PREPARATION OF NUCLEIC ACIDS		
<div style="text-align: center;"><p>5' Probe 3'</p><p>TATCGGCCTCAGGAAGATCGCACTCCAGCCAGCAA AAAABA</p><p> </p><p>ATAGCCGGAGTCCTTCTAGCGTGAGGTCGGTCGAAAGGCCG</p><p>3' M13mp18 5'</p> <p>5' M13 Primer 3'</p><p>TGTAAAACGACGGCCAGT</p><p> </p><p>ACATTTTGCTGCCGGTCA</p><p>3' M13mp18 5'</p></div>		
(57) Abstract		
Disclosed is a composition comprising magnetic particles, each bearing a plurality of oligonucleotides, each oligonucleotide comprising a sequence, acting as a probe, which is complementary to a sequence under investigation, and further comprising a sequence which is non-complementary to the sequence under investigation. Also disclosed is a method of purifying a nucleic acid using the composition of the invention and a method of preparing a nucleic acid.		

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Title Preparation of Nucleic Acids

Field of the Invention

This invention relates to the preparation of nucleic acids and concerns magnetic particles and their use in the preparation of nucleic acids.

Background of the Invention

Currently, a number of large scale DNA sequencing projects are under way. One of the rate-limiting steps in the generation of sequence data is the preparation of high purity DNA templates.

Typically, M13 phage is used to obtain single stranded DNA for sequencing templates. The traditional methods for M13 DNA purification, such as polyethylene glycol (PEG)/phenol procedures (Bankier et al., [1988], in Wu, R. [ed.], Methods Enz. 155, 52-93) allow microgram quantities of template to be produced from millilitre volumes. However, thermally cycled sequencing procedures utilising Taq polymerase (Craxton, [1991], Methods: A Companion to Methods in Enzymology 3, 20-26) only require 200-500ng of template DNA per reaction. Therefore traditional methods produce an excess of template for most purposes, which wastes both time and money.

Some alternative methods of preparing template DNA have been described which employ a microtitre-scale format, and

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thereby reduce waste. For example, one method uses specialised filtration steps (Kristensen et al., [1987], Nucleic Acids Research 15, 5507-5516), whilst another (Smith et al., [1990], Journal of DNA Sequencing and Mapping 1, 73-78) requires the use of PEG/sodium dodecyl sulphate (SDS) with multiple centrifugation steps. Thus these alternative techniques are somewhat cumbersome and impractical.

More recently, a protocol has been described (Alderton et al., [1992], Analytical Biochemistry 152, 304-307) which utilises magnetic particles together with PEG-phage aggregation. A somewhat similar method is disclosed in WO90/06045. This outlines a method of preparing DNA using magnetic beads coated with oligonucleotides, which serve as hybridisation probes, to form a magnetic bead/probe complex. The magnetic beads and any bound nucleic acids can then be magnetically separated from the rest of the sample. However, further improvements in this technology are possible as disclosed below.

Summary of Invention

In one aspect the invention provides a composition comprising magnetic particles, each bearing a plurality of oligonucleotides, each oligonucleotide comprising a sequence, acting as a probe, which is complementary to a sequence under investigation, and further comprising a sequence which is non-complementary to the sequence under investigation.

In another aspect, the invention provides a method of producing the composition defined above, comprising attaching magnetic particles to an oligonucleotide

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comprising a complementary probe sequence and further comprising a non-complementary sequence.

Conveniently, the magnetic particles take the form of beads. Typically these are about 1-5µm in diameter. Suitable magnetic beads are commercially available. For instance, they may be obtained from Dynal or, more preferably, from Promega. Those from Promega are found particularly suitable because they have a pitted surface and therefore a higher surface area, which allows greater amounts of oligonucleotides to be carried by the bead.

'Oligonucleotides' as used herein includes both synthetic and native DNA and RNA sequences of any length. However, the oligonucleotide is preferably single stranded DNA and, conveniently, the complementary probe sequence is substantially 25-45 bases long. The non-complementary sequence is generally 5-15 bases in length, typically about 9 bases long.

Preferably, the non-complementary sequence of the oligonucleotide acts as a 'linker' joining the complementary probe sequence to the magnetic bead. The probe sequence may be attached by either end to the linker oligonucleotide.

The oligonucleotide may be attached to the magnetic bead by conventional techniques. For example, the linker oligonucleotide may conveniently be biotinylated at the end region distal to the end joined to the complementary probe sequence, allowing for ready attachment to streptavidin-coated magnetic beads. Other attachment techniques are known to those skilled in the art.

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The probe sequence can, of course, be selected so as to be complementary to any nucleic acid sequence of interest such that, for example, the sequence of interest may be separated from a complex sample by being bound by the magnetic bead/probe and then separated from the rest of the sample by magnetic attraction.

Thus in another aspect the invention provides a method of separating a nucleic acid sequence from a sample comprising: contacting a complex sample possibly including the sequence of interest with the composition of the present invention; allowing the sequence of interest, if present, to associate with the composition by means of hybridisation with the complementary probe sequence; and separating the composition and any bound nucleic acid sequences from the rest of the sample by magnetic attraction.

Typically the complementary probe sequence comprises an oligonucleotide complementary to the sequence of M13. In a preferred arrangement the invention therefore provides a method of preparing template DNA for sequencing.

Generally the probe sequence is complementary to a region upstream from the M13 -21 universal primer site.

The arrangement described above can confer a number of advantages over prior art methods of purifying nucleic acids. The use of a non-complementary linker sequence reduces the likelihood of steric hindrance between the comparatively bulky magnetic bead and the "target" DNA hybridised to the probe. This allows beads prepared in accordance with the invention to bind far more 'target' DNA than possible hitherto.

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Furthermore, when preparing DNA for sequencing reactions, probe sequences may become separated from magnetic beads of the prior art by shearing and may act as unwanted "false" primers in subsequent sequencing reactions. However, the non-complementary linker oligonucleotide of the invention ensures that, should the oligonucleotides become separated from the beads, they are far less likely to be able to prime chain-extension reactions. It is a preferred feature therefore that the linker sequence should be at the 3' end of the complementary probe sequence, as this further reduces the risk of non-specific priming.

Conventional methods of preparing template DNA from M13 typically involve aggregation of phage particles at room temperature by the use of PEG/NaCl solutions (typically 20% PEG/2.5M NaCl), followed by lysis with SDS to release DNA, which is then bound to the magnetic bead/probe complex. However, it has now been found that advantages accrue from a novel DNA preparation protocol in which M13 particles are first lysed by the action of heat in the presence of a suitable detergent such as SDS. Subsequently, probe/bead complex is added, together with hybridisation buffer containing a suitable polymer such as PEG. Whereas in conventional methods PEG is used to bring about aggregation of the phage particles, in the novel method of the invention it is added after phage lysis to effectively increase the concentration of the target DNA by excluding nucleic acids from the volume taken up by the polymer (Amasino et al., [1992], Analytical Biochemistry 201, 166-169).

Thus in another aspect the invention provides a method of

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preparing a DNA sequence of interest from a complex sample comprising: causing lysis of DNA-containing entities by the action of heat in the presence of a suitable detergent; adding magnetic particles attached to a probe complementary to at least part of the sequence of interest together with hybridisation buffer containing a polymer to cause an increase in the effective nucleic acid concentration; allowing the sequence of interest to hybridise to the complementary probe and then separating the particle/probe complex and any sequences hybridised thereto from the rest of the sample by magnetic attraction.

Any magnetic particles to which are attached an appropriate probe are suitable for performing the method defined above, including those already known in the art. However, it is preferred that the particles used are those according to the present invention.

A suitable detergent is SDS.

The DNA-containing entities are typically phages.

A suitable polymer is PEG. The use of a polymer to increase the effective concentration of nucleic acid allows smaller volumes of reagents to be used than previously, which in turn enables the method to be performed in a microtitre plate or tray. Similarly, the method is particularly simple and efficient as it employs a target sequence-specific purification step.

Thus the invention provides a method of preparing DNA by means of a sequence-specific purification step, capable of being performed in a microtitre plate.

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The ability to perform the method in a microtitre plate makes the method of the invention particularly amenable to automation. Suitable automated apparatus for performing the method of the invention is described in GB9212164.9 (co-pending Application No PCT/GB93/).

The various aspects of the invention may be better understood by reference to the following illustrative example and drawings in which:

Figure 1 shows the base sequence of a suitable probe/linker oligonucleotide;

Figure 2 shows a photograph of gel electrophoresis performed on DNA prepared by the method of the invention; and

Figure 3 shows a portion of sequencing trace

Example

In this particular example, the invention involves an oligonucleotide probe which has been synthesised with a biotin group at the 3' end. The probe is designed to be complementary to a region upstream from the M13 -21 Universal priming site.

In general, single plaques may be grown up in culture, the cells harvested and the supernatant collected and lysed to yield free single strands. The bead/probe complex is then added, and the probe allowed to anneal to the target DNA. Once bound, the bead/probe/template complex can be separated from the rest of the sample using magnetic

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attraction and then washed. The template may then be freed from the bead/probe complex by heating. The procedure is simple and fast. All the post-growth steps can be carried out in microtitre plates with no centrifugation or ethanol precipitations required. In this example, 250 random M13 sub-clones were prepared and sequenced using the method outlined below.

Probe Design

The amount of template recovered depends directly on the design of the probe. The probe must be specific to the target but must not act as a secondary sequencing primer if free probe is left in solution. Consequently, the probe is 41bp in length and binds to a region upstream from the M13 -21 Universal primer site. The probe has a run of several 'A's at the 3' end together with the biotin group. This acts as a linker arm to prevent steric hindrance between the large streptavidin-coated beads and the binding of the probe to the target. Also the high degree of non-complementarity at the 3' end would prevent the free probe from acting as a sequencing primer should it shear from the beads. The design of the probe is shown in Figure 1, ('B' represents Biotin).

The probe THM13.3 was synthesised on an ABI 380B DNA synthesiser on a 1uMole scale. Biotin phosphoramidite was obtained from Amersham U.K. The sequence of the probe was: 5' TAT CGG CCT CAG GAA GAT CGC ACT CCA GCC AGC AAA AAA Biotin A 3' (Seq ID No. 1). Following cleavage from the column, the oligonucleotide was deprotected in ammonia at 55°C overnight. A NAP-10 column was used to purify the crude oligonucleotide.

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Probe/streptavidin bead linkage

Promega nucleotide quality beads were used in this example. 1.2ml of Promega beads were used per 12 samples. The beads were washed in 0.1M NaCl three times using a neodymium-iron-boron permanent magnet to separate the beads from the washing solution. 200ul 0.1M NaCl and 10nmol THM13.3 oligonucleotide were then added to 1.2ml (1.2mg) dry beads and incubated at room temperature for 10 minutes. The beads were then washed 10 times in 0.1M NaCl to remove unbound oligonucleotide. Bead/probe complex was finally taken up in 1.2ml water. Beads may be bound to probe in bulk and stored in storage buffer at 4°C.

M13 Sub-clones

Random M13 sub-clones with 1-2kb inserts were grown up in 2ml 2xTy medium (16g bacto tryptone, 10g yeast extract, 5g NaCl in 1l water) for 5 hours at 37°C. Cells were spun down at 14,000g for 5 minutes and the supernatant transferred to microtitre plates. 400ul supernatant was used from each sample, using two wells, each containing 200ul supernatant (Falcon 3911 MicroTest Flexible Assay Plate.)

Lysis

Prior to addition of supernatant, 10ul of 15% SDS was added to each well using an Eppendorf multidispensing pipette. Phages were lysed by heating the microtitre plate to 70°C for 10 minutes on a Techne PHC-3 cycler.

Annealing

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20ul of hybridisation buffer (20% PEG 8000/2.5M NaCl) was added to each well with a multidispensing pipette; no mixing is required so the same tip may be used for all wells. Also, 20ul bead/probe complex were added to each well, and the dish was incubated at 45°C for 30 minutes.

Wash Steps

The microtitre plate was removed from the cycler and placed on a Dynal MPC-96 magnetic separator. After 30 seconds the supernatant was aspirated, 100ul wash buffer (0.1X SSC) were added to each well and the beads were moved through the wash buffer by repositioning the plate over the magnetic separator three times at intervals of five seconds. This step was repeated a total of three times. Finally, the beads were eluted in 10ul water; using the magnet, beads may be dispersed into this small volume.

Denaturation

The templates were released from the probe by heating the plate to 80°C for 3 minutes. After heating, the beads were concentrated using the magnet and the supernatant was removed to a fresh microtitre plate. Due to evaporation the final total volume was approximately 8ul from each well, or approximately 16ul per DNA template sample.

The efficacy of the technique is illustrated by Figure 2. Four random M13 subclones were grown as described. The cells were harvested and the supernatant (1200ul) was split three ways to provide three identical samples, A, B and C (400ul each). For each sub clone, Sample A was

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prepared using the method outlined previously. Sample B was prepared as outlined but leaving out the hybridisation buffer (20% PEG/2.5M NaCl). Sample C was prepared as outlined but using magnetic beads without a probe attached. The samples were then subjected to agarose gel electrophoresis. This showed that when the PEG is removed there is a dramatic reduction in yield, as expected. When beads are used without probe attached the resulting yield is zero. The difference between this control and the method of Alderton et al., (1992), is that in sample C the phage are lysed before addition of PEG.

Sequencing Results

Once the templates have been purified, as described above, it is possible to immediately perform the sequencing reactions as pre-reaction mixes can be made up and dispensed in advance. The ABI Taq dye primer kit reagents were used. The total volume after recovery of the template from the probe varies from 14-16ul. From this, 2ul has been used in the A/C reactions and 4ul in the G/T reactions. The volume variability does not reduce the quality of the sequence data. All samples were sequenced using the M13 -21 universal primer and Taq polymerase. The reactions were cycled on a Techne PHC-3 dry cycler, each sample being covered with mineral oil. The reaction products were analysed on an ABI 373A DNA sequencer.

In this study, 250 clones taken from the C. elegans cosmids ZK507, ZK512 and K01B6 were prepared using the magnetic probe method and sequenced as described above. The method gave reproducible high quality data which was assembled into current databases. Figure 3 shows a section of a read. The trace shows bases 125-375 from a

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random M13 sub-clone containing a 1-2kb insert. From the sequence data available, it is clear that the procedure is reproducible and not sensitive to fluctuations in template or bead concentrations. Few sub-clones failed to give sequence and overall the results were comparable to the standard PEG/phenol method.

Re-use of Beads

After the preparation of a batch of DNA samples, beads may be re-used as outlined below.

Beads were collected and pooled in a 1.5ml eppendorf tube. The supernatant was removed and the beads were resuspended in 1ml reuse buffer (0.15M NaOH, 0.001% Tween 20) for 1 minute. The supernatant was removed and the procedure repeated. Finally the beads were washed once in storage buffer and taken up in half the original volume of storage buffer (PBS pH 7.5, 0.1% BSA). Beads were then stored at 4°C.

As will be apparent, the preparation steps described above can conveniently be carried out using automated apparatus, for example as described in GB9212164.9 (co-pending international application No PCT/GB93/).

In an alternative method, 50 ul of hybridisation buffer is added to each well, instead of 20 ul as described above, and the volume of beads added to the well reduced slightly to compensate. This enables sufficient template DNA to be obtained from just 200 ul of M13 supernatant (instead of 400 ul as described above). Thus 1 microtitre plate well is sufficient for the analysis of 1 clone, whereas previously 2 wells were required to obtain sufficient

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template DNA from each clone analysed. As a result, twice as many clones can be studied per microtitre plate than was the case previously.

A preferred feature of the method of preparing DNA is to bring about magnetic attraction for the magnetic beads by the use of a "dot magnet". This is a device with the dimensions of a typical microtitre assay plate (generally 130 mm by 85 mm) which comprises an array of rare earth magnets. Preferably the dot magnet comprises an array of 96 such rare earth magnets which are positioned in a matrix 8 x 12 and are separated by about 9mm from each nearest neighbour (ie. substantially the same spacing as that between the wells of a microtitre plate) thus when the sides of the dot magnet are aligned with the sides of a typical microtitre plate, each rare earth magnet is positioned beneath a well in the microtitre plate.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Medical Research Council
(B) STREET: 20 Park Crescent
(C) CITY: London
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): W1N 4AL
(G) TELEPHONE: (071) 636 5422
(H) TELEFAX: (071) 323 1331

(A) NAME: Hawkins, Trevor L.,
(B) STREET: Promega Corp., 2800 Woods Hollow Road
(C) CITY: Madison
(D) STATE: Wisconsin
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 53711-5399

(ii) TITLE OF INVENTION: Preparation of Nucleic Acids

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
(EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TATCGGCCTC AGGAAGATCG CACTCCAGCC AGCAAAAAAA

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SUBSTITUTE SHEET

Claims

1. A composition comprising magnetic particles, each bearing a plurality of oligonucleotides, each oligonucleotide comprising a sequence, acting as a probe, which is complementary to a sequence under investigation, and further comprising a sequence which is non-complementary to the sequence under investigation.
2. A composition according to claim 1, wherein the magnetic particle comprises a bead of 1-5µm in diameter.
3. A composition according to claim 1 or 2, wherein the oligonucleotides are single stranded DNA.
4. A composition according to any of claims 1, 2 or 3, wherein the complementary probe sequence of the oligonucleotides is in the range 25-45 bases long.
5. A composition according to any one of the preceding claims, wherein the complementary probe sequence is complementary to M13.
6. A composition according to any one of the preceding claims, wherein the non-complementary sequence of the oligonucleotides is in the range 5-15 bases long.
7. A composition according to any one of the preceding claims, wherein the non-complementary sequence of the oligonucleotides is biotinylated.
8. A composition according to any one of the preceding claims, wherein the non-complementary sequence of the oligonucleotides acts as a linker between the magnetic

particle and the complementary probe sequence of the oligonucleotides.

9. A composition according to any one of the preceding claims, wherein the non-complementary sequence of the oligonucleotides is at the 3' end of the complementary probe sequence.

10. A method of making the composition of any one of claims 1-9, comprising attaching magnetic particles to an oligonucleotide comprising a complementary probe sequence and further comprising a non-complementary sequence.

11. A method of separating a nucleic acid sequence from a sample, comprising: contacting a complex sample possibly including the sequence of interest with the composition according to any one of claims 1-9; allowing the sequence of interest, if present, to associate with the composition by means of hybridisation with the complementary probe sequence; and separating the particles and any bound nucleic acid sequences from the rest of the sample by magnetic attraction.

12. A method according to claim 11, wherein the sequence of interest comprises a DNA sequence to be determined.

13. A method of preparing a nucleic acid sequence of interest from a complex sample, comprising: causing lysis of nucleic acid-containing entities by the action of heat in the presence of a suitable detergent; adding magnetic particles attached to a probe complementary to at least part of the sequence of interest (hereinafter "particle/probe complex"), together with hybridisation buffer containing a polymer to cause an increase in the

effective nucleic acid concentration; allowing the sequence of interest to hybridise to the complementary probe; and then separating the particle/probe complex and any sequences hybridised thereto from the rest of the sample by magnetic attraction.

14. A method according to claim 13, wherein the detergent is SDS.

15. A method according to claim 13 or 14, wherein the nucleic acid-containing entity is a bacteriophage.

16. A method according to any one of claims 13-15, wherein the nucleic acid is DNA.

17. A method according to any one of claims 13-16 wherein the particle/probe complex is comprised within a composition in accordance with any one of claims 1-9.

18. A method of preparing a nucleic acid by means of a sequence-specific purification step performed in a microtitre plate.

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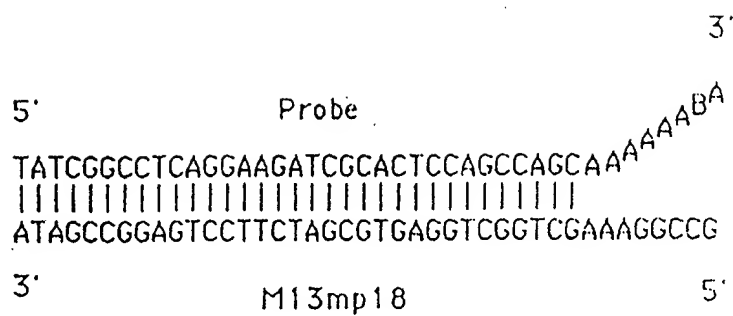


Fig. 1

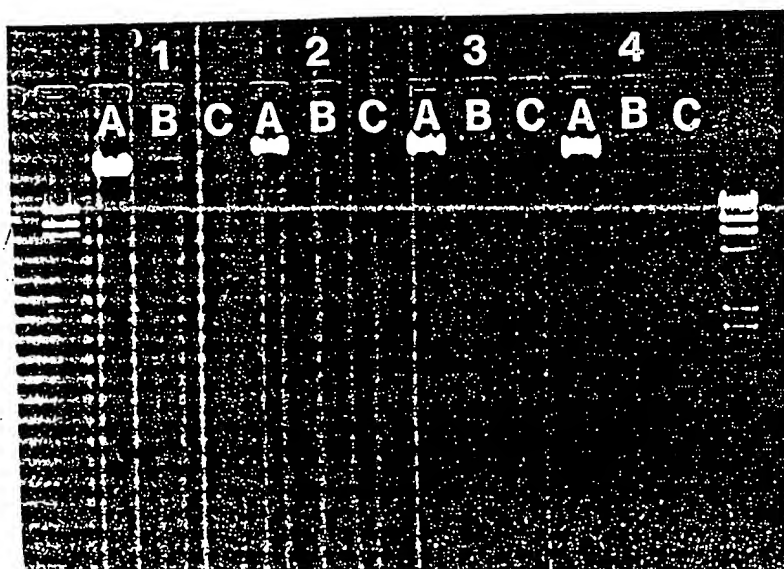


Fig. 2

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/01223

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12Q1/68		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12Q	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,9 006 045 (DYNAL AS) 14 June 1990 cited in the application see the whole document ---	1-18
Y	WO,A,8 605 815 (GENETICS INTERNATIONAL) 9 October 1986 see claims; figure 1 ---	1-18
Y	ANALYTICAL BIOCHEMISTRY vol. 201, February 1992, NEW YORK US pages 166 - 169 ALDERTON ET AL. 'Magnetic bead purification of M13 DNA sequencing templates' cited in the application see the whole document -----	1-18
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
07 OCTOBER 1993		25. 10. 93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		MOLINA GALAN E.

Form PCT/ISA/210 (second sheet) (January 1983)

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9301223
SA 74895

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9006045	14-06-90	AU-B- 640626	02-09-93
		AU-A- 4758690	26-06-90
		EP-A- 0446260	18-09-91
		JP-T- 4501959	09-04-92
		AU-B- 627815	03-09-92
		AU-A- 4758590	26-06-90
		WO-A- 9006044	14-06-90
		EP-A- 0444119	04-09-91
		JP-T- 4501958	09-04-92
		AU-B- 634993	11-03-93
		AU-A- 4759690	26-06-90
		WO-A- 9006042	14-06-90
		EP-A, B 0444120	04-09-91
		JP-T- 4501956	09-04-92
		AU-B- 628442	17-09-92
		AU-A- 4666489	26-06-90
		WO-A- 9006043	14-06-90
		EP-A- 0448609	02-10-91
		JP-T- 4501957	09-04-92
WO-A-8605815	09-10-86	AU-A- 5667186	23-10-86
		EP-A- 0216844	08-04-87

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